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**Article:**

Laing, O., Halliwell, J. and Barbaric, I. (2019) Rapid PCR assay for detecting common genetic variants arising in human pluripotent stem cell cultures. *Current protocols in stem cell biology*. ISSN 1941-7322

<https://doi.org/10.1002/cpsc.83>

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This is the peer reviewed version of the following article: Laing, O., Halliwell, J., & Barbaric, I. (2019). Rapid PCR assay for detecting common genetic variants arising in human pluripotent stem cell cultures. *Current Protocols in Stem Cell Biology*, which has been published in final form at <https://doi.org/10.1002/cpsc.83>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

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# **Rapid PCR Assay for Detecting Common Genetic Variants Arising in Human Pluripotent Stem Cell Cultures**

Running Title: PCR screen for commonly acquired hPSC copy number variants

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## **Significance Statement**

With the increasing use of human pluripotent stem cells (hPSCs) in applications ranging from basic biology to cell replacement therapies, the question of genome integrity of hPSCs has been garnering particular attention. Since hPSCs were first established two decades ago, recurring changes in their genomes have become well documented and recognized as functionally relevant to the behaviour of variant cells in vitro. Regular monitoring of hPSC cultures is pivotal for early detection of variants, but the frequency of screening is hampered by the need for specialist expertise and high costs of currently employed methods. The PCR-based method detailed here provides an effective and rapid tool for routine and high-throughput detection of the most frequently occurring copy number changes in hPSCs.

## **ABSTRACT**

Human pluripotent stem cells (hPSCs) are prone to acquiring genetic changes upon prolonged culture. Particularly common are copy number changes, including gains of chromosomes 1q, 12p, 17q and 20q, and/or loss of chromosomes 10p and 18q. The variant cells harbouring common genetic changes display altered behaviours compared to their diploid counterparts, thus potentially impacting upon the validity of experimental results and safety of hPSC-derived cellular therapies. Hence, a critical quality attribute in hPSC maintenance should include

frequent monitoring for genetic changes arising in cultures. This in turn places large demands on the genotyping assays for detection of genetic changes. Traditional methods for screening cells entail specialized cytogenetic analyses, but their high costs and a lengthy turnaround time make them impractical for high-throughput analyses and routine laboratory use. Here, we detail a protocol for a rapid, accessible and affordable PCR-based method for detection of frequently occurring copy number changes in hPSCs.

**Keywords:** Human Pluripotent Stem Cells (hPSCs), Genetic Changes, Copy Number Variant (CNV), Quantitative Polymerase Chain Reaction (qPCR).

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## INTRODUCTION

Due to their ability to expand extensively in culture and to produce differentiated tissue-specific cell types, human pluripotent stem cells (hPSCs) have become a key tool in disease modelling, drug discovery and regenerative medicine (Takahashi et al., 2007; Thomson et al., 1998). hPSCs can be propagated in a diploid state for long periods of time, but like any other living cells, hPSCs are also subject to genetic alteration that may impact on their phenotype and/or behaviour (Baker et al., 2007; Draper et al., 2004; International Stem Cell Initiative, 2011). The collated data of genetic anomalies in hPSCs demonstrates a spectrum of abnormalities from numerical and structural aneuploidies down to recently detected point mutations in TP53 (Merkle et al., 2017). Genomic imbalances detected in hPSCs exhibit a strong bias towards gains of chromosomal regions, with the most commonly recurring genetic changes encompassing gains of chromosome 1q, 12p, 17q, 20q, and losses of chromosomes 10p and 18q (Baker et al., 2016; Draper et al., 2004; International Stem Cell Initiative, 2011). The non-random nature of these aneuploidies is suggestive of their ability to endow the variant cells with a growth advantage over the wild type cells (Draper et al., 2004). Although the functional consequences of common genetic variants remain poorly characterised, some of the recurrent genetic changes in hPSCs were shown to affect hPSC differentiation (Gertow et al., 2007; Werbowetski-Ogilvie et al., 2009; Fazeli et al., 2011; Ben-David et al., 2014; Varela et al., 2012), reduce the tendency of hPSCs to undergo apoptosis (Avery et al., 2013; Barbaric et al., 2014; Enver et al., 2005) and increase tumorigenicity of undifferentiated hPSCs in vivo (Ben-David et al., 2014). As a corollary of such profound effects of genetic changes on stem

cell behaviour, the presence of variant cells in hPSC cultures could skew experimental results in vitro and potentially hinder progression of stem cell-based therapies into clinic.

To mitigate the risk of variant cells, hPSC cultures should be regularly monitored for their occurrence. Different cytogenetic and molecular methods are available for mutation detection, but given the diversity of the genetic changes arising in hPSC genomes, no single method has the scope to accurately and sensitively detect all types of genetic changes in a single assay. For example, gross cytogenetic changes are easily identifiable from the analysis of metaphases by karyotyping, but small amplifications are often overlooked due to the limited resolution of this method (Simons et al., 2013). In the context of hPSCs genotyping, chromosome 20q copy number variant (CNV) is a particularly frequent, recurrent change that necessitates the use of an alternative or additional technique to karyotyping (International Stem Cell et al., 2011). Examples of methods suitable for CNV detection include Fluorescent In Situ Hybridization (FISH), comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) array platforms. Despite the availability of multiple technologies for genotyping, these approaches can become problematic for routine monitoring of hPSCs as the currently employed cytogenetic and molecular methods are not easily scaled up to accommodate the high-throughput, quick turn-over needs of laboratories that handle many different cultures at any given time. This is especially pertinent to laboratories producing large numbers of hPSC lines or clones, for example when reprogramming somatic cells to hPSCs or producing genetically modified cells using CRISPR/Cas9 technology.

Given that over 50% of all common genomic anomalies in hPSCs involve copy number changes of chromosomes 1q, 12p, 17q, 20q and 18q, we reasoned that screening for those particular genetic changes would allow for a low cost, first-pass screen of hPSC cultures. To this end we have previously designed a targeted panel of primers on commonly amplified regions for a use in a quantitative PCR (qPCR)-based screen (Baker et al., 2016). Our assay is based on relative quantification, whereby the amount of a target locus is first compared to a reference locus within the same sample. For a reference locus we chose a gene on chromosome 4 (RELL1), as copy number changes of chromosome 4 are very rare in hPSCs (Baker et al., 2016; International Stem Cell et al., 2011). The difference in the amount of the target and reference loci are then compared to the difference between the same set of loci in a calibrator sample with a known diploid karyotype.

The whole method as described in this unit entails identification of a calibrator sample (Basic Protocol 1), genomic DNA extraction from hPSCs (Basic Protocol 2), DNA digestion (Basic Protocol 3), running the qPCR (Basic Protocol 4) and data analysis (Basic Protocol 5). Once the calibrator DNA is established it can be used in any subsequent testing, but it should always be included in an assay alongside the test samples. With the calibrator DNA in place, the rest of the assay can be completed within one day. The speed of the protocol, the need for only basic laboratory equipment and a cost of less than £10 per sample, makes this qPCR-based method particularly suitable for rapid first-pass screening of hPSC lines in routine laboratory practice. Comparable to FISH, qPCR detects variant cells when they are present at 10% or more of all cells in a population (Baker et al., 2016).

## **BASIC PROTOCOL 1**

### **Establishing calibrator DNA reference**

The calibrator DNA reference is a sample with diploid copy numbers for target loci, as determined by methods independent of qPCR. Although the calibrator DNA could be extracted from any diploid human cell type, we use hPSCs as a source of our calibrator DNA. Prior to using a DNA sample as a calibrator reference in the qPCR assay, it is important to confirm the diploid status of the cells by karyotyping and Fluorescent In Situ Hybridization (FISH) for chromosome 20q. FISH for 20q must be performed alongside karyotyping, as the 20q CNV often appears below the resolution of karyotyping.

#### **Materials**

Established, growing hPSC culture

1. Choose an early-passage hPSC line that may serve as a calibrator sample. Culture a flask of hPSCs under appropriate culture conditions until the cells are ready for passage.
2. Passage a single culture of growing hPSCs into three parallel cultures (See Support Protocol 1).
3. Two to three days after passaging, process the three parallel cultures as follows:

- i) Harvest the cells from one of the cultures for karyotyping (See Support Protocol 2).
- ii) Harvest the cells from the second parallel culture for detection of chromosome 20q copy number variant by FISH (See Support Protocol 3).
- iii) Harvest the cells from the third culture for genomic DNA extraction (see Basic Protocols 2 and 3).

Note: the same procedure can be used to validate the presence of a copy number change in cells. If trisomy encompasses the minimal regions targeted by our panel of primers on chromosomes 1q, 12p, 17q, 20q or if monosomy of 18q is detected, the DNA of variant cells can be used as a positive control in the qPCR assay.

## **SUPPORT PROTOCOL 1**

### **Passaging hPSCs**

The following procedure should be carried out in a laminar flow hood under sterile conditions.

#### **Materials**

Vitronectin (Cat. No. A14700, Life Technologies)

Dulbecco's Phosphate Buffered Saline (PBS), without calcium chloride and magnesium chloride

hPSC culture

Essential 8 (Cat no. A1517001, Life Technologies)

ReLeSR (Cat no. 05873, StemCell Technologies)

T12.5 cell culture flasks

1. Prepare three T12.5 flasks by coating them with vitronectin (diluted 1:100 in PBS), for at least an hour at room temperature. After an hour, remove vitronectin and add 1.5mL pre-warmed Essential 8 culture medium per flask.

2. Remove the medium from the hPSCs growing in a T12.5 flask on vitronectin in Essential 8 and wash once with PBS.
3. Add 1mL ReLeSR into the flask and swirl the flask to ensure that ReLeSR covers all of the cells on the bottom of the flask. Aspirate ReLeSR within one minute. Aspirating the liquid will leave behind only a thin layer of ReLeSR coating the cells. Leave the flask at room temperature for 4min.
4. Tap the side of the flask with one hand to facilitate detachment of colonies/ clumps of cells. Add 1.5mL Essential 8 culture medium onto cells and pipette gently once or twice to ensure that all the cells detach from the flask. Distribute the cells into the three T12.5 flasks with Essential 8 by pipetting 0.5mL of cells per flask.

Note: a longer incubation with ReLeSR and/or repeated pipetting after the addition of the medium will result in smaller clumps and dissociation to increased numbers of single cells, which in turn may negatively impact on the survival of cells post-passaging. The conditions suggested here are provided as a general guideline, but ideally should be optimised for each hPSC line and each operator.

5. Culture the cells at 37°C in 5% CO<sub>2</sub> with daily replenishing of the culture medium.

## **SUPPORT PROTOCOL 2**

### **Processing of hPSCs for karyotyping**

#### **Materials**

KaryoMAX Colcemid Solution in PBS (Cat. no. 15212012, ThermoFisher Scientific)

Dulbecco's Phosphate Buffered Saline (PBS), without calcium chloride and magnesium chloride

TrypLE Express Enzyme (Cat.no. 11528856, Fisher Scientific)

DMEM/F12 (D6421-500ML, Sigma-Aldrich)

0.0375M KCl, prewarmed to 37°C

Fixative (3 parts methanol : 1 part acetic acid, v/v)

15ml tubes

Laboratory centrifuge

1. Add 0.1µg/ml KaryoMAX Colcemid solution in culture medium to the flask of hPSCs and incubate at 37°C, 5% CO<sub>2</sub> for 2-4 hours.
2. Remove the medium with colcemid and wash once with PBS.
3. Add 1mL of TrypLE Express Enzyme per flask and incubate at 37°C for 3 minutes.
4. Add 4mL basal medium (DMEM/F12) and transfer to a 15mL tube, making sure all of the cells have been collected from the flask.
5. Centrifuge the cells at 270 g for 8 mins at room temperature.
6. Remove the supernatant, taking care not to disturb the cell pellet.
7. Re-suspend the pellet in pre-warmed 0.0375M KCl hypotonic solution and incubate for 10 min at room temperature.
6. Pellet the cells by centrifuging them at 270 g for 8 mins at room temperature.
7. Add 2mL fixative (3 parts methanol : 1 part acetic acid, v/v) onto the cells, in a drop-wise manner.

It is important that the fixative is added slowly, in a drop-wise manner, whilst continuously agitating the tube with cells. If fixative is added too quickly, it may lyse the cells.

**Pause point:** The fixed cells can now be stored at 4 °C prior to sending off to a cytogenetics laboratory for preparation of metaphase spreads and karyotyping.

### SUPPORT PROTOCOL 3



## Processing of hPSCs for FISH analysis

### Materials

Dulbecco's Phosphate Buffered Saline (PBS), without calcium chloride and magnesium chloride

TrypLE Express Enzyme (Cat.no. 11528856, Fisher Scientific)

DMEM/F12 (D6421-500ML, Sigma-Aldrich)

0.0375M KCl, prewarmed to 37°C

Fixative (3 parts methanol : 1 part acetic acid, v/v)

15ml tubes

Laboratory centrifuge

1. Remove the medium from the flask of hPSCs and wash once with 3mL PBS.
2. Remove PBS and add 1mL of TrypLE Express Enzyme onto cells. Incubate at 37°C for 3 mins.
3. Add 4mL basal medium (DMEM/F12) and transfer to a 15mL tube, making sure all of the cells have been collected from the flask.
4. Centrifuge the cells at 270 g for 8 mins at room temperature.
5. Remove the supernatant, taking care not to disturb the cell pellet.
6. Add 1mL of 0.0375M potassium chloride pre-warmed to 37°C.
7. Centrifuge the cells at 270 g for 8 mins at room temperature.
8. Remove the supernatant, taking care not to disturb the cell pellet.
9. Add 2mL fixative (3 parts methanol : 1 part acetic acid, v/v) onto the cells, in a drop-wise manner.

It is important that the fixative is added slowly, in a drop-wise manner, whilst continuously agitating the tube with cells. If fixative is added too quickly, it may lyse the cells.

**Pause point:** The fixed cells can now be stored at 4 °C prior to sending off to a cytogenetics laboratory for FISH analysis.

## **BASIC PROTOCOL 2**

### **Genomic DNA extraction**

An essential requirement for qPCR-based detection of copy number variants is the extraction of good quality genomic DNA from hPSC cultures. We commonly use QIAGEN DNeasy Blood & Tissue Kit, although alternative commercial kits for DNA extraction are also available.

#### **Materials**

hPSC cultures or hPSC pellets stored at -20°C

DMEM/F12 (D6421-500ML, Sigma-Aldrich)

Dulbecco's Phosphate Buffered Saline (PBS), without calcium chloride and magnesium chloride

Qiagen DNeasy Blood & Tissue Kit (Cat. no. 69506, Qiagen)

Ethanol, Absolute

Rubber policeman or equivalent tools for scraping off the cells

15ml tubes

Laboratory centrifuge

Pipettes covering a range of 1-1000µl

Nuclease-free tips

Microcentrifuge capable of  $\geq 20,000g$

Microcentrifuge tubes 1.5ml

Vortex mixer

NanoDrop (Thermo Fisher) or similar spectrophotometer

#### **DNA Extraction**

1. Remove the culture medium from the flask containing hPSCs and replace it with 3mL basal medium, such as DMEM/F12.
2. Scrape the cells off the bottom of the flask using a rubber policeman.
3. Transfer the cells into a 15ml tube and pellet them by centrifuging at 160 g for 3 min.
4. Carefully remove the supernatant without disturbing the cell pellet.

Alternatively, cells can be harvested by enzymatic methods, such as trypsin, TrypLE or accutase, and counted prior to pelleting. This may be useful if the cultures are particularly confluent and yielding numbers of cells higher than  $>2.5 \times 10^6$ . According to the manufacturer's instructions of the QIAGEN DNeasy Blood and Tissue DNA extraction kit, the columns can take up to  $5 \times 10^6$  cells, but in our experience, pellets of more than  $2.5 \times 10^6$  cells can overload the column, resulting in inefficient washing steps and ultimately a lower purity of extracted DNA.

**Pause point:** Cell pellets can be placed at  $-20^{\circ}\text{C}$  and the DNA extraction continued at a later date. Upon removal of the pellets from the freezer, allow the pellets to thaw at room temperature for up to five minutes. Dislodge the pellets by firmly tapping on the outside of the tube.

5. Resuspend the cell pellet in 200 $\mu$ l PBS and follow the QIAGEN DNeasy Blood and Tissue Quick protocol according to the manufacturer's instructions, with the following amendments:

Pipette 40µl of Buffer AE per every 500,000 cells harvested, onto the centre of the purification column. Incubate the column with the Buffer AE at room temperature for 10 minutes prior to spinning it for 1 minute at  $\geq 6000$  g to elute the DNA.

### **DNA Quantification**

1. Vortex the eluted DNA samples briefly before determining the quantity and quality of the extracted DNA by spectrophotometry.

Note that the DNA samples with an A<sub>260</sub>/A<sub>280</sub> ratio <1.80 are not considered sufficiently pure and therefore should be either further purified or discarded.

### **DNA Storage**

1. Store the DNA samples at 4°C for up to 2 months or at -20°C for up to 2 years.

## **BASIC PROTOCOL 3**

### **DNA Digestion**

Digesting extracted genomic DNA using a restriction enzyme improves PCR efficiency and robustness. Our protocol uses EcoRI as we confirmed that none of the amplicons in our qPCR panel contain an EcoRI site. DNA (1µg) digestion is performed in a 50µl reaction, followed by a 1:1 dilution in nuclease-free water. The resulting 10ng/µl solution of digested DNA is then ready for use in the qPCR assay. We perform restriction digests in a thermal cycler, but conventional heat blocks or water baths can be employed to the same effect.

### **Materials**

DNA ( $\geq 200$ ng/µl)

Nuclease-free water

10X FastDigest Buffer (Cat. no. B64, ThermoScientific)

FastDigest EcoRI (Cat. no. FD0275, ThermoScientific)

8-strip PCR tubes with lids

Pipettes covering a range of 1-1000 $\mu$ l

Nuclease-free tips

Thermal cycler or a heat block

1. Label the PCR tubes appropriately and add 1 $\mu$ g of sample DNA.

If the Qiagen DNeasy Blood & Tissue Kit was used for DNA extraction, the DNA concentration should be  $\geq 200$  ng/ $\mu$ l, because AE elution buffer should not make up more than 20% of the total reaction volume.

2. Add nuclease-free water up to 44 $\mu$ l.
3. Add 5 $\mu$ l 10X FastDigest Buffer to each reaction.
4. Add 1 $\mu$ l of FastDigest EcoRI to each reaction and seal lids.

The use of FastDigest EcoRI speeds up the process of restriction digest. However, conventional EcoRI with a longer incubation time can also be used to digest the DNA.

5. Run reactions in a thermal cycler using the following conditions:

37°C, 30mins

80°C, 5mins

6. Add 50 $\mu$ l nuclease-free water to each completed reaction to yield a 10ng/ $\mu$ l solution of digested DNA.

## **BASIC PROTOCOL 4**

## qPCR setup

The first step in the qPCR set up is determining the number of reactions to be included on a 384-well plate. In addition to test DNA samples, a calibrator sample, a positive DNA control (if available) and a no-template control for each of the target/reference loci should be included. Each sample – locus combination should be tested in triplicate wells. To reduce the amount of pipetting and improve accuracy, PCR reagents are combined into master mixes. As different primer pairs are used for testing different loci, separate master mixes must be made for each of the five target loci (1q, 12p, 20q, 17q and 18q) and for the internal reference locus (4p) (**Table 1**). The master mixes should be aliquoted into the appropriate wells of the 384-well plate before adding the DNA, to prevent cross-contamination of samples. The use of multi-dispense pipettes speeds up the process of aliquoting the master mixes and DNA samples, and reduces pipetting errors.

## Materials

Nuclease-free water

TaqMan® Fast Universal PCR Mix (2X) (Cat.no. 4366072, ThermoFisher Scientific)

10 $\mu$ M forward and reverse primer solutions (sequences detailed in **Table 1**)

Roche Life Sciences UPL Probes #12, #13, #25, #44, #60 (Roche)

EcoRI-digested DNA at a concentration of 10ng/ $\mu$ l

Pipettes covering a range of 1-1000 $\mu$ l

Nuclease-free tips

1.5ml microcentrifuge tubes

Vortex mixer

Multi-dispense electronic pipettes covering a range of 2-100 $\mu$ l

384 well standard PCR plate

Adhesive PCR plate seal

Centrifuge with plate carriages

qPCR thermal cycler (e.g. QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Life Technologies))

The following procedure should be carried out on ice, with all solutions kept on ice, in order to minimise evaporation from wells and to minimise Taq polymerase activity prior to placing the reaction in the thermal cycler.

1. Determine the number of reactions to be included on a 384 well plate. The DNA samples should include the test samples, a calibrator DNA sample and no template control for each of the master mixes. If available, also include positive control samples with known trisomies or monosomies of target loci. Each reaction should be run in triplicate. An example of a 384 well plate layout has been provided in **Figure 1**.
2. Prepare qPCR master mixes for each locus as indicated in **Table 2**. Components of the master mix should be added in the sequence listed. Crucially, the light sensitive hydrolysis probe should be added last to minimise the light exposure. Mix each component thoroughly by vortexing prior to use. A surplus of three or more reactions per master mix should be made to allow for loss of solutions to plastic ware and pipetting error.
3. Mix each of the master mixes thoroughly by vortexing and aliquot 8 $\mu$ l per well of a 384 well plate according to the plate map from step 1.

We use a multi-dispense pipette to speed up the aliquoting process, with a new pipette tip used for each of the master mixes.

4. Vortex the DNA samples and using a multi-dispense pipette, add 2µl of 10ng/µl EcoRI-digested DNA per well.

Use one pipette tip per three replicate wells, and then discard the tip and use a new one prior to pipetting the DNA into next set of three wells containing a different master mix. This is necessary to prevent possible cross-contamination of master mixes.

5. Seal the plate using the adhesive plate sealing film.

Special attention should be taken to ensure that every well and the edges of the plate are completely sealed. Any gaps between sealing film and well will lead to evaporation of the reaction mixture, thereby altering concentrations of reagents and affecting the results.

6. Briefly spin the plate to ensure the reaction mixtures have collected in the bottom of the wells.

7. Load the plate into the qPCR Thermal cycler and run the following profile:

Hold Stage: 50°C 2 minutes

Denaturation: 95°C 10 minutes

40 Cycles at 95°C 15seconds, 60°C 1 minute

All ramp stages are at 1.6°C/s.

## **BASIC PROTOCOL 5**

### **Data analysis**

#### **Glossary**

**Calibrator** - a sample with diploid copy numbers for target loci, as determined by methods independent of qPCR.



**Internal reference locus** – a locus within the genomic DNA which is expected to have a stable copy number. It is measured alongside the target locus in a sample and used to normalise the target locus copy numbers. For a reference locus we chose a gene on chromosome 4 (RELL1), as copy number changes of chromosome 4 are very rare in hPSCs.

**Target locus** – locus or gene of interest. In our assay we are determining the copy numbers of loci 1q, 12p, 17q, 18q and 20q, as these are the most commonly observed copy number changes in hPSCs.

**Cq (cycle quantification)** - the number of cycles in a qPCR run at which fluorescence from PCR amplification exceeds the background fluorescence (Bustin et al., 2009).

**Relative quantification of copy numbers** – a quantification approach used to determine the copy numbers of a target locus based on a comparison to a calibrator sample (Livak & Schmittgen, 2001).

1. When the qPCR run is completed, obtain the quantification cycle (Cq) values for each of your replicate reactions using the auto-baseline settings of the QuantStudio 12K Flex Software (or equivalent if using an alternative qPCR machine) and export them into an Excel file for further processing.

Note; Cq values are defined as the number of cycles at which fluorescence from PCR amplification exceeds the background fluorescence. Although the QuantStudio 12K Flex Software reports Ct (threshold cycle) values, MIQE guidelines proposed the standardisation of nomenclature in the interest of better experimental practice in the use of qPCR (Bustin et al., 2009). Hence, from herein we will use the term Cq values instead of the Ct values.

Example of raw data Cq values from a qPCR run:

Sample Name	Target Name	Cq
Calibrator	4P	24.365
Calibrator	4P	24.358
Calibrator	4P	24.327
Sample 1	4P	23.979
Sample 1	4P	23.977
Sample 1	4P	24.172
Calibrator	1Q	24.435
Calibrator	1Q	24.383
Calibrator	1Q	24.341
Sample 1	1Q	23.357
Sample 1	1Q	23.559
Sample 1	1Q	23.506

Technical replicates: calibrator sample, reference locus

Technical replicates: unknown sample, reference locus

Technical replicates: calibrator sample, target locus

Technical replicates: unknown sample, target locus

Note: As a quality control step, check that the Cq values of your technical replicates do not differ by more than 0.3. A large variation in Cq values may be a result of inaccurate pipetting and warrants a repeat of the experiment.

- For each DNA sample, calculate the Cq average for the reference locus in your sample by averaging values of the three technical replicates:

$$\text{Cq average}_{(4p \text{ reference locus of the sample})} = \text{Sum [Cq of the technical replicates for the 4p reference locus of the sample]} / \text{number of technical replicates}$$

$$\text{Cq average}_{(4p \text{ reference locus of the sample})} = (23.979 + 23.977 + 24.172) / 3 = 24.04$$

- Calculate dCq of a test locus within a DNA sample by subtracting the calculated average Cq value of the internal reference locus (4p) from the Cq values from each replicate well (n) of the target locus, as follows:

$$dCq_{(\text{target locus of the sample, replicate } n)} = Cq_{(\text{target locus of the sample, replicate } n)} - Cq_{\text{average}_{(4p \text{ reference locus of the sample)}}$$

$$dCq_{(\text{target locus of the sample, replicate 1})} = 23.357 - 24.04 = -0.683$$

$$dCq_{(\text{target locus of the sample, replicate 2})} = 23.559 - 24.04 = -0.481$$

$$dCq_{(\text{target locus of the sample, replicate 3})} = 23.506 - 24.04 = -0.534$$

4. Calculate the Cq average for the reference locus in your calibrator by averaging values of the three technical replicates:

$$Cq_{\text{average}_{(4p \text{ reference locus of the calibrator})}} = \text{Sum [Cq of the technical replicates for the 4p reference locus of the calibrator]} / \text{number of technical replicates:}$$

$$Cq_{\text{average}_{(4p \text{ reference locus of the calibrator})}} = (24.365 + 24.358 + 24.327)/3 = 24.35$$

5. Calculate dCq of the target locus within the calibrator by subtracting the average Cq value of the internal reference locus (4p) from the Cq values from each replicate well of the target locus for the calibrator sample:

$$dCq_{(\text{target locus of the calibrator, replicate } n)} = Cq_{(\text{target locus of the calibrator, replicate } n)} - Cq_{\text{average}_{(4p \text{ reference locus of the calibrator)}}$$

$$dCq_{(\text{target locus of the calibrator, replicate 1})} = 24.435 - 24.35 = 0.085$$

$$dCq_{(\text{target locus of the calibrator, replicate 2})} = 24.383 - 24.35 = 0.033$$

$$dCq_{(\text{target locus of the calibrator, replicate 3})} = 24.341 - 24.35 = -0.009$$

6. Calculate the dCq average for the target locus in your calibrator by averaging values of the three technical replicates:

$$\text{dCq average}_{(\text{target locus of the calibrator})} = \text{Sum [dCq of the technical replicates for the target locus of the calibrator]} / \text{number of technical replicates}$$

$$\text{dCq average}_{(\text{target locus of the calibrator})} = (0.085 + 0.033 - 0.009)/3 = 0.109$$

7. Determine the ddCq value for each replicate by subtracting the average dCq of the calibrator sample for a given locus from the dCq of the same locus in the test sample:

$$\text{ddCq}_{(\text{target locus of the sample, replicate n})} = \text{dCq}_{(\text{target locus of the sample, replicate n})} - \text{dCq average}_{(\text{calibrator target locus})}$$

$$\text{ddCq}_{(\text{target locus of the sample, replicate 1})} = -0.683 - 0.109 = -0.792$$

$$\text{ddCq}_{(\text{target locus of the sample, replicate 2})} = -0.481 - 0.109 = -0.59$$

$$\text{ddCq}_{(\text{target locus of the sample, replicate 3})} = -0.534 - 0.109 = -0.643$$

8. Calculate the relative quantity of the test locus in the sample by raising 2 to the power of -ddCq:

$$\text{Relative quantity}_{(\text{target locus of the sample, replicate n})} = 2^{-\text{ddCq}_{(\text{target locus of the sample, replicate n})}}$$

$$\text{Relative quantity}_{(\text{target locus of the sample, replicate 1})} = 2^{-(-0.792)} = 1.73$$

$$\text{Relative quantity}_{(\text{target locus of the sample, replicate 2})} = 2^{-(-0.59)} = 1.51$$

$$\text{Relative quantity}_{(\text{target locus of the sample, replicate 3})} = 2^{-(-0.643)} = 1.56$$

9. Determine the copy number of the test locus within the sample of interest by multiplying the relative quantity value by 2:

$$\text{Copy number}_{(\text{target locus of the sample, replicate } n)} = 2 * \text{Relative quantity}_{(\text{target locus of the sample, replicate } n)}$$

$$\text{Copy number}_{(\text{target locus of the sample, replicate } 1)} = 2 * 1.73 = 3.46$$

$$\text{Copy number}_{(\text{target locus of the sample, replicate } 2)} = 2 * 1.51 = 3.02$$

$$\text{Copy number}_{(\text{target locus of the sample, replicate } 3)} = 2 * 1.56 = 3.12$$

10. Finally, calculate the average of the technical replicates (n) for each target locus within a sample:

$$\text{Average copy number}_{(\text{target locus of the sample})} = \frac{\text{Sum} [\text{Copy number}_{(\text{target locus of the sample, replicate } n)}]}{\text{number of technical replicates}}$$

$$\text{Average copy number}_{(\text{target locus of the sample})} = (3.46 + 3.02 + 3.12) / 3 = 3.2$$

11. To determine the cut-off points for classifying samples as containing a variant population, calculate the standard deviation of the copy numbers obtained for each of the target loci within the calibrator. Multiply the standard deviation by a factor of three. In our experience, this calculation usually yields a number of around 0.2. Thus, samples with copy numbers higher than ~2.2 or less than ~1.8 can be classed as containing a mosaic variant population with a trisomy or monosomy of a locus, respectively (**Figure 2**).

## SUPPORT PROTOCOL 4

### The use of the qPCR assay in determining the size of the amplified region

Primer sets designed to span the length of a chromosome make it possible to adapt the qPCR assay to measure the length of an amplicon. Support Protocol 4 details how the assay can be adapted to measure the amplicon length on chromosome 20q.

1. The primers (**Table 3**) were designed to target genes spanning the length of the q arm of chromosome 20 (**Figure 3A**). Each primer set is separated by approximately 100-200 kb.
2. The PCR assay should be performed as described in Basic Protocol 4, but substituting the primers used in **Table 1** with those listed in **Table 3**. Remember to include the calibrator sample and, if available, the positive control sample.
3. Data analysis should be performed using the protocol listed in Basic Protocol 5. Copy number values above or below three times the standard deviation are classified as containing a mosaic population. The approximate amplicon length can be determined based on the positive values (**Figure 3B**).

## SUPPORT PROTOCOL 5

### Primer Design and Testing

One of the major advantages of the qPCR-based method for detection of genetic changes is its flexibility, meaning that the range of loci tested can be easily expanded by incorporating new primer pairs into the test panel to screen for the copy number of any gene or sequence in the genome. The process requires preliminary *in silico* screening of primer pairs to predict specificity and lack of self-complementarity in primers. This is followed by *in vitro* screening of candidate primer pairs initially for specificity via melting curve analysis to ensure primers amplify only one product. Finally, amplification efficiencies of primers are determined by generating standard curves.

## Materials

Nuclease-free water

SYBR® Green JumpStart™ Taq ReadyMix™ (Cat.no. S4438, Sigma-Aldrich)

10μM forward and reverse primer solutions

EcoRI-digested DNA at a concentration of 10ng/μl

EcoRI-digested DNA at a concentration of 50ng/μl

TaqMan® Fast Universal PCR Mix (2X) (Cat.no. 4366072, ThermoFisher Scientific)

Roche Life Sciences UPL Probes (Roche)

Pipettes covering a range of 1-1000μl

Nuclease-free tips

1.5ml microcentrifuge tubes

Vortex mixer

Multi-dispense electronic pipettes covering a range of 2 -100μl

384 well standard PCR plate

Adhesive PCR plate seal

Centrifuge with plate carriages

qPCR thermal cycler (e.g. QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Life Technologies))

## Primer Design

1. Retrieve a genomic sequence of a gene/locus of interest by using a human genome browser, such as the Ensembl ([https://www.ensembl.org/Homo\\_sapiens/Info/Index](https://www.ensembl.org/Homo_sapiens/Info/Index)).
2. Copy an intronic region of sequence from within the gene and paste the sequence into Roche Life Sciences Assay Design Centre

([https://www.lifescience.roche.com/en\\_gb/brands/universal-probe-library.html#assay-design-center](https://www.lifescience.roche.com/en_gb/brands/universal-probe-library.html#assay-design-center)).

3. Uncheck the “Automatically select an intron spanning assay” box and click “Design primers” to obtain a panel of candidate primers and corresponding hydrolysis probes.
  4. Screen amplicons of the top four candidate primer pairs for the absence of an EcoRI restriction site: GAATTC. Proceed only with the primer pairs whose expected PCR amplicons do not harbour the EcoRI restriction site.
  5. To check the specificity of the primers, copy candidate primer sequences into the NCBI Primer-Blast database (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to screen for self-complementarity and specificity. Prior to screening the primers, adjust Primer Pair Specificity Checking Parameters to the genomes for selected organisms option.
  6. Choose a primer pair determined to be specific by the above in silico method and generate 10 $\mu$ M working stocks as detailed in the reagents and solutions section.
- Given the low cost of primers, to speed up the testing we typically purchase at least three different primer pairs per locus of interest.

### **Melting Curve Analysis**

Melting curve analysis is used to verify the specificity of the PCR reaction by confirming the presence of a single PCR product, and absence of non-specific products and/or primer dimers. It is based upon the property of the DNA double helix to denature with an increase in temperature, with the temperature at which denaturing occurs affected by the PCR amplicon sequence and length. Melting curve analyses employ fluorescent dyes, such as SYBR Green I dye, which bind non-specifically to DNA duplex but not to single stranded DNA. Thus, during melting curve analysis the fluorescence of a completed PCR reaction is continually monitored over a gradual rise in temperature. A sharp drop in fluorescence indicates the melting temperature ( $T_m$ ) of the product. The thermal cycler software typically produces melting curves as part of the menu options and plots melting peaks. Specific primers will amplify only one product, corresponding to a solitary peak in the resulting graph, whereas the presence of non-specific amplicons and primer dimers is evident as multiple peaks.

The following procedure should be carried out on ice, with all solutions kept on ice, in order to minimise evaporation from wells and to minimise Taq polymerase activity prior to placing the reaction in the thermal cycler.



1. Determine the number of reactions to be included on a 384-well plate. Each primer pair should be run in triplicate wells with a DNA sample as well as a no-template negative control.
2. Prepare SYBR Green Master mixes for each locus as indicated in **Table 4**. Mix each component thoroughly by vortexing prior to use. A surplus of one or more reactions per master mix should be made to allow for loss of solutions to plastic ware and pipetting error.
3. Mix each of the master mixes thoroughly by vortexing and aliquot 18µl per well of a 384 well plate.

We use a multi-dispense pipette to speed up the aliquoting process, with a new pipette tip used for each of the master mixes.

4. Vortex the DNA samples and using a multi-dispense pipette, add 2µl of 10ng/µl EcoRI-digested DNA or no-template control per well.

Use one pipette tip per three replicate wells, and then discard the tip and use a new one prior to pipetting the DNA into next set of three wells containing a different master mix. This is necessary to prevent possible cross-contamination of master mixes.

5. Seal the plate using the adhesive plate sealing film.

Special attention should be taken to ensure that every well and the edges of the plate are completely sealed. Any gaps between sealing film and well will lead to evaporation of the reaction mixture, thereby altering concentrations of reagents and affecting the results.

6. Briefly spin the plate to ensure the reaction mixtures have collected in the bottom of the wells.
7. Load the plate into the qPCR thermal cycler and run the following profile:

#### **PCR Amplification**

Hold Stage: 50°C 2 minutes

Denaturation: 95°C 10 minutes

40 Cycles: 95°C 15seconds, 60°C 1 minute

#### **Melting Curve Analysis**

Heating: 95°C 15seconds

Cooling: 60°C 1 minute

Denaturation: 0.05°C/second to 95°C

The final denaturation step is carried out under continuous fluorescence monitoring to determine the  $T_m$  of the amplified product(s).

8. Export melting profiles using the machine software and check for the absence of a second peak on the melting peak plot. A single peak indicates amplification of one product and therefore specificity of the primer pair.

### Assessment of PCR amplification efficiency

The relative quantification method used in this unit relies on similar amplification efficiencies of different primers employed in the protocol. In theory, the amount of PCR product should double in each cycle of the PCR reaction, and such a reaction is considered a 100% efficient. However, in practice, the amplification efficiency often deviates from the ideal and needs to be assessed empirically. Amplification efficiency can be taken as the proportion of target molecules within a reaction that are amplified per cycle of PCR. Efficiencies are commonly expressed as percentages and are determined by generating standard curves. This entails plotting the  $C_q$  values of reactions containing decreasing concentrations of DNA over a 1:5 serial dilution against the logarithmic values of DNA concentrations. This should yield a linear plot, the slope of which is used in determining the amplification efficiency.

1. Serially dilute EcoRI digested DNA 1:5 in nuclease-free water to generate five samples of decreasing DNA concentration ranging from 50ng/ $\mu$ l to 0.08ng/ $\mu$ l.
2. Set up a qPCR reaction for the candidate primer pairs as per **Basic Protocol 4**. Each DNA concentration should be treated as a new sample. For this assay there is no requirement for a specific calibrator or positive control. A negative no-template control should still be included.
3. Run the reaction as per the temperature profile in **Basic Protocol 4**.
4. Upon completion of the reaction, export  $C_q$  values and plot them against the concentration of DNA on a logarithmic scale with a line of best fit between datasets.
5. Calculate the slope of the line of best fit and determine the efficiency of amplification for each primer pair as a percentage using the following equation:

$$\text{Efficiency} = -1 + 10^{-(1/\text{slope})} * 100\%$$

6. A primer pair for each locus should be selected based on efficiency closest to that of the internal reference locus.

Primer efficiencies ranging from 90-115% are acceptable. If no candidate efficiencies fall within these limits then further primer pairs should be designed and tested.

## REAGENTS AND SOLUTIONS

### TE Buffer

10 mM Tris-HCl, pH 8.0

0.1 mM EDTA

### 0.0375M KCl

3,75 mL 1M KCl

96,25 mL ddH<sub>2</sub>O

### 10 µM primer mixes

Primer sequences for each of the target and reference loci are listed in **Table 1**. Primers purchased in a lyophilised form are resuspended in TE Buffer or ddH<sub>2</sub>O to a final concentration of 100µM. Working solutions of primers are made by combining the forward and reverse primer for each locus, with each primer at a final concentration of 10µM:

50µl 100µM forward primer

50µl 100µM reverse primer

400µl ddH<sub>2</sub>O

Working stocks can be stored for up to 3 months at 4°C, whereas stock solutions should be stored at -20°C for long-term storage.

## COMMENTARY

### Background Information

The possibility of culture-acquired genetic variants invalidating the outcomes of experimental results or safety and efficacy of clinical therapies, has brought sharply into focus the need for regular screening of cultures. Although an early identification of variants is pivotal in preventing them from overtaking the cultures, at the practical level, the frequent screening using methods such as karyotyping, FISH or CGH arrays, is hampered by the need for specialised expertise and high costs involved. The qPCR-based method described in this unit, based on that originally described by Baker et al. (2016), screens for five of the most common chromosomal abnormalities reported in hPSCs. The primary advantages of this technique lie in its accessibility, low cost (less than £10/sample) and fast turnaround. Furthermore, it has the ability to detect very small amplifications and deletions that fall below the resolution of karyotyping. The chromosome 20q CNV is one such frequently arising change that necessitates the use of methods alternative to karyotyping. Indeed, in an International Stem Cell Initiative study over 20% of 120 cell lines tested had a gain of 20q CNV, but as many as 22 of them were missed by karyology (International Stem Cell et al., 2011). The sensitivity of the qPCR assay to detect mosaic abnormalities in hPSC cultures is also comparable to other commonly employed methods, such as karyotyping and FISH, allowing detection of variants when they are present at 10% or more of all cells within a culture (Baker et al., 2016). However, it should be noted that the qPCR assay described here has a number of inherent drawbacks. First and foremost, this method can only detect copy number changes in the regions targeted with primers. Hence, any other copy number changes outside of the examined loci will go undetected. Also, the results obtained by this method are indicative only of copy numbers of the specific genes screened and bare no reference to the nature of the genetic abnormality. For instance, a copy number of three could indicate a gain of a whole chromosome as is the case

for chromosomes 1 and 12 in S6-GFP hPSC sample shown on **Figure 2**. Alternatively, the copy number of three could also represent a tandem duplication of a very small amplicon, as is the case for chromosome 20q gain in TC113 E12-RFP hPSC clone (**Figure 2**). A further possibility is the occurrence of an isochromosome. Classically, acquisition of an isochromosome entails an unbalanced structural rearrangement of arms of a chromosome whereby either a p or q arm of a chromosome is lost and replaced with a q or p arm respectively, resulting in a chromosome comprised 2 p arms or 2 q arms. Distinguishing between a whole chromosome gain, an isochromosome or a CNV is theoretically possible by qPCR, but it requires the use of primers on the p arm to reveal if the p arm is also amplified (a whole chromosome gain), there is no change in p arm copy (CNV gain) or p arm has only one copy (isochromosome of 20q). Finally, the qPCR assay is also unable to detect structural variants that show no net gain or loss in genetic material, i.e. balanced translocations. Hence, we recommend that the qPCR assay detailed here should be used as a rapid and simple first-pass screen to frequently monitor hPSC cultures or to examine the presence of most common genetic changes in clones following reprogramming or genetic modifications. For more detailed genome-wide analyses, additional methods such as SNP arrays or next generation sequencing methods should be employed.

### **Critical Parameters**

The success of the assay relies on the access to an appropriate calibrator DNA sample, as all test samples are compared to the calibrator. If the calibrator DNA is of poor quality or is not diploid for all analysed loci and for the internal reference gene, the results for the test samples will be skewed accordingly. For example, data normalised to a calibrator sample harbouring a 10% population of cells with 1q trisomy could designate a test sample harbouring 20% population of cells with 1q trisomy as “normal”. Hence, in addition to a calibrator DNA, a positive control with a known trisomy or monosomy of target regions should ideally be

included within each qPCR run. The inclusion of positive control samples serves as an objective quality control of the qPCR run, whereby the assay is designated as failed if the positive control copy number results deviate from the ones expected.

### Troubleshooting

Problem	Possible cause	Solution
Results for a given locus consistently high/low across samples	Calibrator DNA and/or working primer stocks degraded	Thaw fresh aliquots of calibrator gDNA and primer mixes.
	Calibrator DNA includes population of CNV cells	Use a different calibrator sample
	Poor primer design	Consider using different primer pairs for the locus in question
High C <sub>q</sub> variability between triplicate wells	Pipetting Error	Ensure that the pipette tip reaches the bottom of each well prior to dispensing. Avoid touching other parts of the plate with a loaded tip.
	Poorly mixed qPCR master mix or DNA sample	Vortex all components thoroughly prior to dispensing into wells of the plate.
	Reaction evaporation due to ineffective sealing of plate	Take care to ensure every well on the PCR plate is sealed appropriately with the film.
Implausibly low copy number results (<1)	Low quality sample DNA, possibly due to degradation of DNA sample or co-purification of molecules inhibitory to PCR during the DNA extraction.	Use nuclease-free water and solutions during DNA extraction. Store DNA samples at 4°C for short periods of time or at -20°C for long term storage. If freezing the samples, avoid repeated freeze-thaw cycles. If the quality of DNA is low due to poor extraction, consider counting the cells to ensure that the columns are not overloaded beyond their capacity.

### Expected Results

Typically, the results of this qPCR assay yield copy number values between two and three for chromosomes 1q, 12p, 17q and 20q, and between one and two for chromosome 18q. A copy number value of three for a particular locus indicates that the whole cell population tested is trisomic for that locus. On the other hand, a copy number value higher than ~2.2 and lower than ~2.8 would indicate that a cell population is mosaic for the presence of variant cells. Although trisomies are the most common abnormalities in hPSCs, multiple gains of 20q CNV have also been noted. For example, we have detected a population of cells with four copies of 20q in TC113 E6-RFP hPSCs (**Figure 2**).

### **Time Considerations**

The whole qPCR-based assay described here, from DNA extraction and digestion, to qPCR set up and PCR run to interpretation of qPCR results, can be completed within a day. However, this assumes that the calibrator DNA has been obtained and validated as diploid. Given that the validation means include methods alternative to qPCR (such as karyotyping, FISH, CGH or SNP arrays), the whole process of establishing the calibrator DNA can take several weeks. It is advisable to make larger batches of the calibrator DNA, as once obtained, it can be used for any further testing.

### **ACKNOWLEDGEMENTS**

This work was partly funded by the UK Regenerative Medicine Platform, MRC reference MR/R015724/1, and partly by the European Union's Horizon 2020 research and innovation programme under grant agreement No. 668724.

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- Baker, D., Hirst, A. J., Gokhale, P. J., Juarez, M. A., Williams, S., Wheeler, M., ... Barbaric, I. (2016). Detecting Genetic Mosaicism in Cultures of Human Pluripotent Stem Cells. *Stem Cell Reports*, 7(5), 998–1012. <https://doi.org/10.1016/j.stemcr.2016.10.003>

The protocol described in this unit is based on that originally described in the above paper.

## FIGURE LEGENDS

### Figure 1

Example of a 384-well plate layout for the qPCR assay. Samples, including calibrator (blue), positive control (red), and no template negative control (black) are arranged in columns of three replicate wells. Master mixes for each locus are arranged in rows in 2 repeating modules at the top and bottom of the plate.

### Figure 2

QPCR analysis of the copy numbers at loci 1q, 12p, 17q, 18q and 20q in four different hPSC lines. Green lines on graphs denote cut-off points above or below which the samples can be deemed positive for the presence of the variant cells. S6-GFP is the positive control sample and exhibits trisomies at all loci with the exception of 18q. TC113 G7 shows a copy number of 2 at all screened loci, as confirmed by karyology. TC113 E12-RFP was originally a clonal population with trisomy of 20q only however, over time in culture has acquired trisomy of 12p in a population of cells. TC113 E6-RFP has gained a triplication at locus 20q.

### Figure 3

The application of the qPCR assay in the determining the length of the 20q CNV.

A. Schematic representation of the chromosome 20q amplicon, indicating gene names where the primers for screening the amplicon length are located.

B. Assessment of the chromosome 20q CNV length in the cell line NCRM1. The 20q CNV length is assessed based on the copy number change. The overall amplicon length in this line is approximately 480kb, with the breakpoint situated between the genes TPX2 and MYLK2.

## TABLES

**Table 1.** Details of the target loci included in the qPCR assay.

Target locus	Gene (location) Accession Number	Primer Sequences (forward and reverse)	UPL Probe Number	Amplicon Length (bp)
4p	RELL1 (4p14) NC_000004.12	tgcttgctcagaaggagctt tgggttcaggaacagagaca	#12	64
1q	MDM4 (1q32.1) NC_000001.11	gccccagacctaataatcaat tcggtatgacagcaatgtctct	#13	76
12p	DPPA3 (12p.13.31) NC_000012.12	cgtagcgtcggtgcatca tcctttttaccgttctgaca	#60	60
17q	TK1 (17q23.2- q25.3) NC_000017.11	ggtgacagctgcttacagcttag actggttgccaccttctcag	#60	64
18q	PHLPP1 (18q21.33) NC_000018.10	tcaagcattgcccttagctt gccttaaagcatcacttccatc	#25	88
20q	BCL2L1 (20q11.21) NC_000020.11	tctgcagaaggctacccta tgctgtgtctaaagaccttctcat	#44	75

**Table 2.** qPCR master mix volumes

Component	Final concentration	Volume/reaction ( $\mu$ l)
Nuclease Free water		2.8
TaqMan® Fast Universal PCR Mix (2X)	1X	5
Forward and Reverse Primer Mix 10 $\mu$ M	0.1 $\mu$ M	0.1
Universal Probe Library hydrolysis probe 10 $\mu$ M	0.1 $\mu$ M	0.1 $\mu$ l

**Table 3.** Primers and UPL probes used to determine the length of the chromosome 20q CNV region.

Target locus	Gene (location) Accession Number	Primer Sequences (forward and reverse)	UPL Probe Number	Amplicon Length (bp)
4p	RELL1 (4p14) NC_000004.12	tgcttgctcagaaggagctt tgggttcaggaacagagaca	#12	64
20q	DEFB115 (20q11.21) 31,257,664 NM_001037730.1	tcagcctgaacattctggtaaa cacttgctctttccccaactc	#14	76
20q	REM1 (20q11.21) 31,475,272 NM_014012.5	ccccttttctcactccacaa tctgcagggggagaagtaca	#46	64
20q	BCL2L1 (20q11.21) 31,664,452 NC_000020.11	tctgcagaaggctacccta tgctgtgtctaagacctttcat	#44	75
20q	TPX2 (20q11.21) 31,739,101 NM_012112.4	cccccaaatcaggcctac ttaagcaaaatccaggagtcaa	#35	59
20q	MYLK2 (20q11.21) 31,819,375 NC_000020.11	ggtcaggagaacccagagtg gtctcccagggcacttcag	#16	62
20q	XKR7 (20q11.21) 31,968,002 NM_033118.3	gtgtcttaccggggctctatc gcctggaagggtgtgcagta	#3	59
20q	TM9SF4 (20q11.21) 32,109,506 NM_014742.3	taatggagccaatgccagta caaaaccagtttctgtgccttt	#45	59
20q	ASXL1 (20q11.21) 32,358,062 NM_015338.5	gagtgtcactgtggatgggtag ctggcatatggaaccctcac	#13	59

**Table 4.** SYBR® Green master mix volumes

Component	Final concentration	Volume/reaction (µl)
Nuclease Free water		7.8

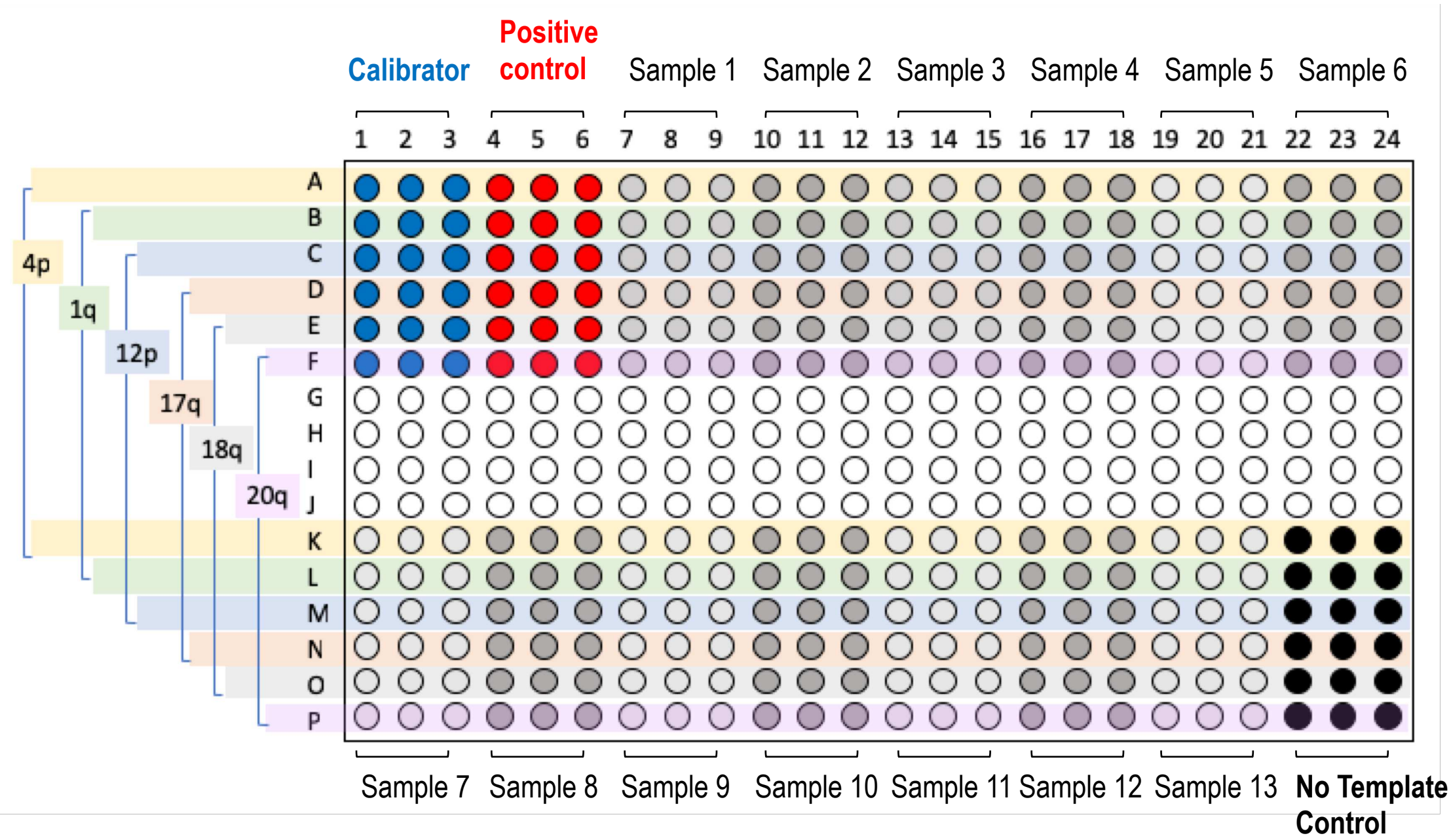
SYBR® Green	1X	10
JumpStart™ Taq		
ReadyMix™		

Forward and Reverse	0.1 µM	0.2
Primer Mix 10 µM		

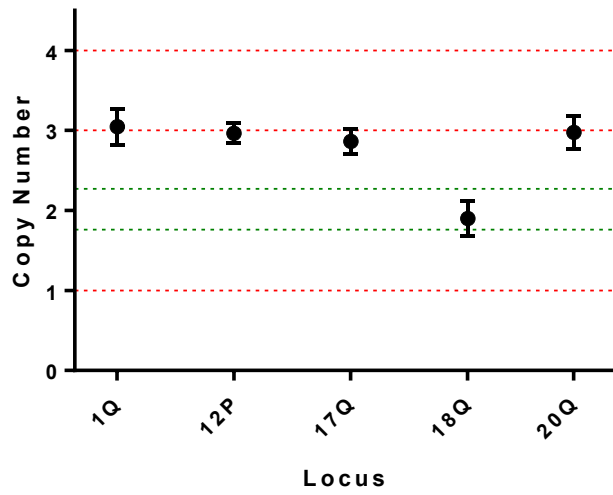
UNCORRECTED PROOF

Figure 1.

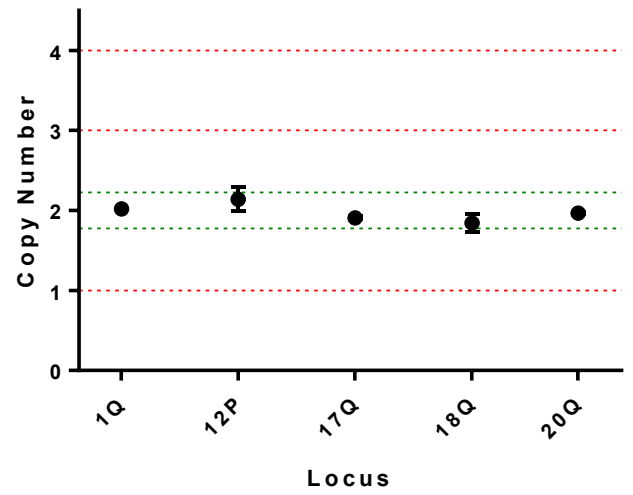
Master  
mixes for  
loci tested



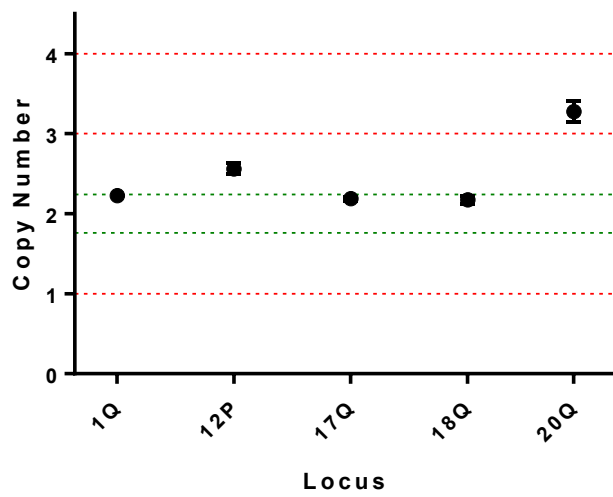
**S6-GFP +VE**



**TC113 G7**



**TC113 E12-RFP**



**TC113 E6-RFP**

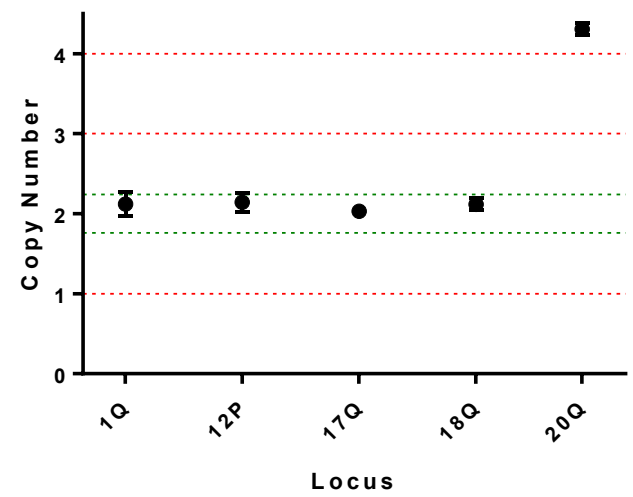
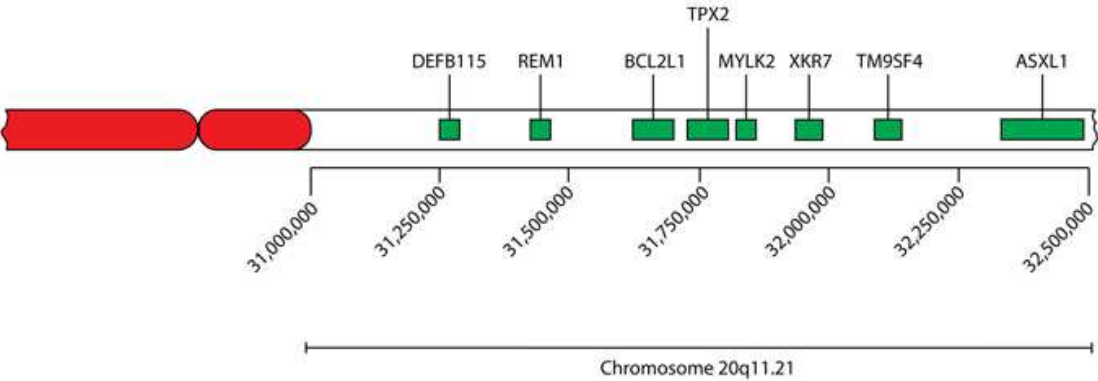


Figure 3

A



B

